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The purpose of this proposal is to develop a "next generation" representational difference analysis protocol that uses transcribing nuclear DNA. This protocol should detect all transcribe genes aberrantly transcribed during the progression of breast cancer. Genes, for example, that produce strictly nuclear RNA should be isolated by this protocol. Our first specific aim was to isolate transcriptionally active nuclear DNA from human breast cancer cells. Features that distinguish transcribing chromatin from bulk chromatin are unfolded nucleosomes with thiol-reactive histone H3 and highly acetylated H3. A new method using Sulfolink column chromatography has been developed to isolate transcribing chromatin with thiol-reactive H3 from T47D5 breast cancer cells. Further, we are in the process of developing an alternate procedure for the isolation of transcriptionally active chromatin by a chromatin immunoprecipitation approach. Here we use anti-acetylated H3 antibodies to immunoprecipitate highly acetylated H3 that is cross-linked to DNA fragments. We show that both methods will isolate transcriptionally active chromatin.

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4. TABLE OF CONTENTS

5. INTRODUCTION

A. TRANSCRIPTIONALLY ACTIVE GENES AND NUCLEAR MATRIX

One of the exciting realizations made in recent years is that the nucleus is highly organized [1-5]. Transcribed genes are found in discrete foci, with about 300 transcription foci being observed in HeLa cells [6]. The nuclear matrix is the foundation on which this organization is built, providing a dynamic structural support from which nuclear processes such as DNA replication and transcription occur [2,7,8].

The nuclear matrix has a role in organizing nuclear DNA. Nuclear DNA is arranged into loop domains, with the base of the loop being anchored to the nuclear matrix. The DNA region at the base of the loop is called the matrix attachment region. Nuclear DNA is packaged into nucleosomes. The nucleosome consists of two each of the four core histones, H2A, H2B, H3 and H4 around which is wrapped DNA. The core histones are arranged as a $[H3\text{-}H4]_2$ tetramer flanked on either face with H2A-H2B dimers. Histone H1 binds to the DNA that joins the nucleosomes together. With H1 most chromatin loops are in a condensed configuration. This higher order chromatin packaging makes the genes within this loop essentially invisible to the transcription machinery. Transcriptionally active genes are found in DNAase I-sensitive, decondensed chromatin loops that are accessible to transcription factors, transcription machinery and restriction endonucleases [9-11].

Transcribed and nontranscribed sequences are precisely compartmentalized within the nucleus, with transcriptionally active chromatin being attached to the nuclear matrix [12-14]. Actively transcribed chromatin regions are immobilized on the nuclear matrix by multiple dynamic attachment sites [13]. The transcription machinery, specific transcription factors, and nuclear enzymes (e.g., histone acetyltransferase and deacetylase) are thought to mediate the dynamic attachments between transcriptionally active chromatin and the nuclear matrix [15-19].

B. NUCLEAR MATRIX AND BREAST CANCER

Pathologists have long appreciated that irregular nuclear appearance is the signature of a malignant cell [20,21]. Changes in nuclear shape correlate with progression of breast cancer [22]. Since the nuclear matrix, the structural framework of the nucleus, governs

the shape of the nucleus, this observation suggests that the structure of a breast cancer cell's nuclear matrix is being altered during progression of the disease. The nuclear matrix is composed of protein and RNA. Changes in either nuclear matrix protein proteins and/or RNA could contribute to alterations in nuclear matrix structure of breast cancer cells, which, in turn, would affect gene expression [23]. We have recently published several papers showing the changes in nuclear matrix proteins found in breast cancer cells and tumors that are estrogen receptor (ER) positive or ER negative [24,25].

Approximately 75% of nuclear RNA cont[illegible]sequences not found to a significant extent in the cytoplasm as stable poly $(A)^+$ cytoplasmic mRNA. Cells depleted of nuclear RNA display a disruption in chromatin and nuclear matrix organization [26-28], suggesting that hnRNA (heterogeneous nuclear RNA) may have a structural role in organizing the higher order structure of chromatin. In support of this, Hogan and colleagues have identified the Drosophila *Hsr-omega-n* RNA transcript as an unprocessed RNA molecule that is distributed evenly within the nucleus and not found in the cytoplasm [29]. Similarly, an RNA transcript referred to as *XIST,* which does not contain an open reading frame to encode for protein, is found almost exclusively in the nucleus [30,31]. This transcript has a functional role in the inactivation of the X chromosome in females [32,33].

The current model of development of breast tumorigenesis is a series of morphological evolving from normal epithelium to increasingly abnormal cellular modifications including hyperplasia, atypical hyperplasia, in-situ carcinoma, and finally metastatic carcinoma [34]. Identification of aberrantly expressed genes by detection of differentially expressed mRNAs is a powerful approach to understand the molecular basis for progression breast cancer. However, mRNA based approaches will not detect aberrantly transcribed genes whose products are strictly nuclear. Our knowledge of this class of transcribed genes is minimal. Cancer related, aberrant transcription of genes coding for strictly nuclear RNAs may lead to alterations in nuclear matrix structure, leading to transformations in nuclear structure, function and to genetic instability [35].

C. TRANSCRIPTIONALLY ACTIVE NUCLEOSOMES

In mammalian cells, H3 is the only histone with a cysteine [36]. This cysteine residue at position 110 in H3 is buried in the interior of most nucleosomes and is not available to thiol reactive reagents. Nucleosomes associated with transcribing DNA sequences are the only exceptions. These nucleosomes are structurally altered, with exposed H3 cysteines [37]. Nucleosomes associated with newly replicated chromatin do not have exposed H3 thiol groups [38]. The transcription process is required to form thiol reactive nucleosomes [39]. Nucleosomes associated with RNA polymerase II transcribed genes in mammalian cells (including human cells) cease to be thiol reactive when RNA polymerase II transcription is arrested with α-amanitin [40]. Another feature of transcriptionally active nucleosomes is that they are associated with highly acetylated core histones [40-42].

Dr. Allfrey and colleagues exploited the unique thiol reactive property of transcriptionally active nucleosomes to isolate transcribing chromatin by mercury affinity column chromatography [37,39,40,43-47]. Genes transcribed by RNA polymerase I, II and III are associated with the thiol reactive nucleosomes, with approximately 80% of the mercury-bound nucleosomes originating from genes transcribed by RNA polymerase II [44]. In studies with chicken immature erythrocyte chromatin, we found that both transcription elongation and highly acetylated histones are needed to observe the thiol reactive nucleosome [48]. Thus, to isolate transcriptionally active chromatin, we have two "handles", one is thiol reactive H3 of the unfolded, transcribing nucleosome and the other is the highly acetylated H3.

D. ACETYLATED HISTONES AND TRANSCRIPTIONALLY ACTIVE CHROMATIN

Highly acetylated histones are associated with transcriptionally active and competent (chromatin that is ready for transcription) chromatin [41,42]. We have learned that the enzymes catalyzing histone acetylation, histone acetyltransferase and histone deacetylase, are transcriptional coactivators and repressors, respectively. Thus, histone acetylation of a gene locus is initiated by the recruitment of a histone acetyltransferase/coactivator by transcription factors bound to the gene's promoter or enhancer [42,49]. Clearly, there is an intimate relationship between transcription and

histone acetylation. The reason for this is that acetylation of histones results in the relaxation of chromatin, facilitating the loading of transcription factors onto promoters and elongation [50,51]. Several coactivators involved in steroid hormone signaling are histone acetyltransferases, e.g., CREB-binding protein, steroid receptor coactivator 1, and amplified in breast cancer 1 protein [42]. The ER, a transcription factor essential to the proliferation of hormone-dependent breast cancer cells, recruits these coactivators only when it is associated with its ligand estradiol [42,52,53]. In the isolation of chromatin with acetylated histones, the technique called **CHIPs** (chromatin immunoprecipitation) has become popular [54-56].

Proteins that are repressors (e.g., Mad-Max) often recruit a complex containing the histone deacetylase [42,57,58]. The recruited histone deacetylase deacetylates histones, resulting in the condensation of the chromatin fiber. When the ER is associated with the antiestrogen, hydroxytamoxifen, it will recruit a complex containing the histone deacetylase [59-61]. There is evidence that ER positive breast tumors that are resistant to tamoxifen treatment are deficient in the proteins that allow the recruitment of the histone deacetylase complex [61].

Both histone acetyltransferases and deacetylases are located at transcriptionally active chromatin sites. Thus, histone acetylation at these sites is dynamic. We found that the unfolded, thiol reactive nucleosome structure is maintained for as long as the histones remain highly acetylated. Once the histones are deacetylated, the nucleosomes fold to a thiol unreactive state [48]. There are several inhibitors that can inhibit the histone deacetylase, including sodium butyrate and trichostatin A [48].

The knowledge that histone acetylation and deacetylation events are linked to transcriptional regulation combined with evidence that the pattern of histone acetylation is altered in some cancers, suggests that histone acetylation is an important factor in cancer development [58,62-64]. Aberrant recruitment of histone acetyltransferases or deacetylases can lead to inappropriate gene expression.

E. RESEARCH OBJECTIVES

The goal of this research was to establish a protocol to isolate transcriptionally active chromatin from human breast cancer cells. Once this objective was completed, we were going to design a "next generation" representational difference analysis (**RDA**) protocol that uses transcribing DNA. This protocol should detect **all** transcribed genes aberrantly transcribed during the progression of breast cancer. Our strategy was to exploit features of transcriptionally active chromatin to isolate nuclear DNA of transcribed genes. In our statement of work we had set out three tasks as follows:

Task 1 Months 1-15 Tissue culture of T47D5 human breast cancer cells

Task 2 Months 2-15 Develop protocol to isolate transcribing chromatin from T47D5 nuclei. Isolate "active" DpnII fragmented DNA.

Task 3 Months 2-15 Preparation of plasmids containing DNA sequences that are transcriptionally active or repressed in T47D5 cells. Southern blot analysis of "active" DpnII fragmented DNA with radiolabelled DNA probes.

In this report we show the progress that we have made in achieving these tasks. In the isolation of transcriptionally active chromatin, we used two methods, organomercury affinity column chromatography and Sulfolink capture chromatography, to isolate thiol reactive unfolded nucleosomes of transcriptionally active chromatin. This method has been successful. However, problems were encountered when we attempted to isolate transcriptionally active chromatin from the nuclear matrix. Concurrently, we have been establishing the CHIPs methodology, which will be used to isolate transcriptionally active chromatin by its highly acetylated histones.

6. BODY OF REPORT

A. EXPERIMENTAL METHODS

i. Cell Culture

Human breast cancer cell line T47D5 (ER positive and estrogen dependent) was grown in DMEM (GIBCO) medium supplemented with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin sulfate (100 mg/ml), and 5% glucose in a 37°C humidified incubator with 5% CO_2. Two batches of T47D5 cells, passage number P18 and P98, were continually grown and used in the experiments. In some experiments, the cells were incubated in the presence of 10 mM sodium butyrate, a histone deacetylase inhibitor. After the cells covered 80% of the area in the dish, the medium was removed and replaced with fresh medium, containing 10 mM sodium butyrate. The cells were incubated at 37°C for 60 to 120 min, and washed with phosphate buffer, and harvested. The cell pellets were used immediately or stored at -80°C.

ii. Restriction Enzyme Digestion of Nuclei from Breast Cancer Cells

Approximately 1 x 10^7 T47D5 breast cancer cells were washed in ice-cold TNMT buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1% thiodigycol). The cells were then resuspended in 5 ml of TNMT buffer, homogenized and incubated on ice for 5 min. Triton X-100 was added to the cells to a final concentration of 0.5% (v/v) and the cells were passed through a 22 g needle 3 times. The nuclei were rinsed two times with TNMT buffer and 2 A_{260} units were resuspended in 1 ml of digestion buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 5 mM sodium butyrate) containing 1 mM phenylmethysulfonyl fluoride (PMSF). The nuclei were digested for 3 h at 37°C with either 2 or 5 U of enzyme (DpnII or Msp1) per μg of DNA. The DNA from the digested nuclei was isolated by ethanol precipitation and run on a 0.8% agarose gel.

iii. Fractionation of Breast Cancer Chromatin

Chromatin fractionation was done as described previously [12,65]. Two different buffer systems (Buffer B and TNM buffer) were used to optimize the chromatin fractionation for

chromatography. In brief, nuclei were isolated in RSB buffer (10 mM NaCl, 10 mM Tris-Cl, pH 7.5, 3 mM $MgCl_2$, 1 mM PMSF, 5 mM sodium butyrate), and resuspended in buffer B (10 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.35 M sucrose, 0.1 mM PMSF, 5 mM sodium butyrate) to 20 A_{260}/ml. Micrococcal nuclease was added to a final concentration of 15 units/ml. After incubation at 37°C for 10 min, digested nuclei were collected by centrifugation at 12000 g for 10 min. The supernatant (S0) was saved. The pellets were resuspended in 10 mM EDTA (pH 7.5) and left on ice for at least 30 min to release chromatin fragments into solution. The soluble fraction (SE) and insoluble fraction (PE) were separated by centrifugation at 12000 g for 10 min.

For some studies TMN buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 0.3 M sucrose, 1 mM PMSF) instead of buffer B was used. The procedure to prepare chromatin fractions using TMN buffer is the same as the Buffer B system. The only difference is that the nuclei were prepared and resuspended in TMN buffer for micrococcal nuclease digestion.

iv. Organomercury Chromatography

Organomercury chromatography was performed as described previously [48]. Before loading onto the column, the chromatin fraction S_E or S_0 was dialysed against Buffer A (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 25 mM NaCl, 5 mM sodium butyrate, 5 mM EDTA, 0.1 mM PMSF). The chromatin fraction was applied to the organomercurial column (Affi-Gel 501, Bio-Rad) in the dark.

v. Sulfolink Coupling Gel Column Chromatography

Sulfolink Coupling Gel (Pierce) consists of immobilized iodoacetyl on a cross-linked agarose support. All of the procedures were carried out in the dark. The Sulfolink agarose beads were washed with five volumes of coupling buffer (50 mM Tris-HCl, pH 8, 5 mM EDTA). The chromatin fraction S0, SE or PE was suspended in coupling buffer and then loaded onto a Sulfolink column (20 A_{260} of chromatin/ 1 ml of Sulfolink gel). The column was incubated at room temperature with rotation for 15 min, then without rotation for another 15 min. The unbound fraction was collected. The column was washed with five

volumes of coupling buffer. Five volumes of 0.8 M NaCl in coupling buffer was passed through the column. Five volumes of 1.2 M NaCl in coupling buffer and 2 M NaCl in 5 M urea (or 2 M NaCl in coupling buffer in some experiments) were subsequently passed through the column to elute chromatin bound to the column. In some experiments, 1 ml of either chromatin fraction S0 or SE (4 A_{260}) in coupling buffer was applied to 0.2 ml of Sulfolink agarose in a microcentrifuge tube. For some experiments the chromatin-loaded column was washed with 0.8 M NaCl in coupling buffer, and then the bound DNA was released by digesting the beads with proteinase K, followed by the phenol/isoamyl alcohol extraction and ethanol precipitation of the DNA.

To check the specificity of Sulfolink column, a cysteine block experiment was done. The Sulfolink beads were washed with five volume of coupling buffer. The beads were incubated with 50 mM cysteine in coupling buffer, and untreated beads were incubated with equal volume of coupling buffer at room temperature for 15 min with rotation, then for 15 min without rotation. After centrifugation to remove the buffer, the beads were washed with five volume of coupling buffer. Chromatin fraction SE was added into the treated and untreated Sulfolink beads, and incubated as described above. The aliquots of unbound fractions were collected, and the OD_{260} (optical density at 260 nm) measured. The ratios of OD_{260} for unbound from the treated and untreated columns were calculated.

vi. DNA Preparation and Southern Blotting

DNA preparation was done as described previously [12]. DNA concentration was determined by the diphenylamine assay [12]. DNA was prepared from fractions by RNase A and proteinase K digestion then extracted with phenol and chloroform [12]. Ten mg of DNA was loaded onto a 0.8% agarose gel. After electrophoresis, DNA was transferred onto Hybond N+ (Amersham) nylon membrane, and hybridized with ^{32}P labelled probes. The cloned probes were: pHsp70myc, which contains the human c-*myc* exons 1 and exon 3 (obtained from Dr. B. Shiu, University of Manitoba); pHGER5, which consists of the human ER exon3 (obtained from Dr. L. Murphy, University of Manitoba); pS2, which contains the human ps2 cDNA sequence (obtained from Dr. B. Shiu); R10, which contains the human apolipoprotein B 5' matrix attachment region sequence (from Dr. G. Delcuve,

Cangene). Inserts from the plasmids were processed as follows: GHER5/ EcoR I and Sal I to yield a 2.8 kb fragment; pS2/ Pst I to yield a 350 bp fragment; RH10/ Xba I to yield a 2.5 kb fragment; Hsp70myc/ BamH I to linearize plasmid. The DNA probes were labelled with ^{32}P α-dCTP using the RadPrime DNA Labelling System (BRL).

vii. Protein Preparation and Western Blotting

The histones were extracted from fractions with 0.4 N sulphuric acid and TCA precipitated as described [12]. Electrophoresis of proteins was performed using SDS (sodium dodecyl sulfate) -15% polyacrylamide and AUT (acetic acid urea) -15% polyacrylamide gels as described [66]. Anti-acetylated H3 and anti-acetylated H4 antibodies were generously supplied by Dr. D. Allis and were used to analyse acetylated histones in Western blotting.

viii. Isolation and Incubation of Chicken Erythrocytes

Chicken immature erythrocytes were isolated from adult White Leghorn chickens as described in a previous study [12] except that the collection buffer did not contain sodium butyrate, and chicken erythrocytes were incubated with sodium butyrate for 30 min.

ix. Erythrocyte Chromatin Fractionation

The fractionation of chromatin was performed in the presence of 1 mM PMSF as previously described [12]. In brief, nuclei (50 A_{260} units/ml) were digested with micrococcal nuclease (15 units/ml for 10 min at 37°C), collected by centrifugation and then resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA to release the chromatin fragments from the nuclei. The sample was then centrifuged, and the supernatant (SE) made to 150 mM NaCl and centrifuged in order to separate the insoluble chromatin fraction (P150) from the salt-soluble chromatin (S150).

x. Assessment of Formaldehyde Cross-linking Efficiency

We have devised a novel strategy to determine the efficiency of formaldehyde cross-linking. PMSF was added at each step in the procedure to a 1 mM final concentration. Nuclei from immature chicken erythrocyte were digested with micrococcal nuclease as

previously described, resuspended in a Hepes buffer (10 mM Hepes, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM sodium butyrate), treated with 1% formaldehyde for either 0, 5, 10, 14, or 60 min, and washed in RSB buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM sodium butyrate). The cross-linked cells were resuspended in a lysis buffer (5 M urea, 2 M guanidine-HCl, 2 M NaCl, 200 mM potassium phosphate, pH 7.5), sonicated for 3-30 sec intervals at 155 watts to fragment DNA to approximately 0.5 to 2.5 kb, and then centrifuged to remove insoluble proteins. A_{260} measurements of the supernatant were recorded to determine the percentage of nuclear DNA released by the sonication. The supernatant was applied to pre-equilibrated hydroxyapatite (HAP) (1 g HAP for every 4 mg DNA), and the mixture was incubated with constant rotation at 4°C for 1h. Proteins not bound to the HAP through their cross-linked DNA sequences and RNA were removed by washing the slurry three times in lysis buffer. The HAP was resuspended in lysis buffer and incubated at 68°C for 6 h. The slurry was centrifuged, and the supernatant was dialyzed against double distilled water, lyophilized, and resuspended in 8 M urea. Protein concentrations of each sample were determined using the BioRad protein assay and BSA as a standard. Ten μg of protein from each sample was electrophoresed on a SDS-15% polyacrylamide gel and transferred to nitrocellulose overnight at 30 V at 4°C. Immunochemical detection of the highly acetylated H3 isoform was then performed as described below.

xi. Immunoprecipitation of Highly Acetylated Histone H3

Immunoprecipitations utilizing an antibody that recognizes only tri- and tetra-acetylated isoforms of histone H3 were performed according to a procedure by Dedon *et al.* [67]. The S150 fraction isolated from the chromatin fractionation procedure was cross-linked with 1% formaldehyde (v/v) for 5 min at room temperature. We chose only this fraction because it is highly enriched in highly acetylated H3 isoforms and transcriptionally active chromatin. The reaction was stopped by adding glycine to a final concentration of 0.125 M. The sample was then dialysed against 10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, 10 mM sodium butyrate, and concentrated with PEG 8000 (Fischer Scientific; Nepean, Ontario). 100 μg of the concentrated S150 was made up to 250 mM NaCl, 25 mM Tris-HCl

(pH 7.5), 5 mM Na_2EDTA, 1% Triton X-100, 0.1% SDS), 0.5 µg/ml leupeptin and 1 mM PMSF (SB250 buffer) in a final volume of 500 µl. Sixty µl of Protein A agarose beads (1:1 bead to SB250 buffer slurry) (Pierce) was added to the sample for 15 min at 4°C. This reduced the amount of proteins able to non-specifically bind to the protein A agarose in the presence of the primary antibody. The protein A agarose was then removed, and the anti-acetylated histone H3 antibody was added to the pre-cleared sample in a ratio of 1:200. The sample was incubated overnight at 4°C with constant rotation. Protein A agarose beads (60 µl of a 1:1 bead to SB250 buffer slurry) were added to the sample, and the sample was incubated for 3 h at 4°C with constant rotation. Following this, the protein A beads were washed in 1 ml of 1X RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate (SDC), 1% NP-40), 1 ml of high-salt wash (500 mM NaCl, 1.0% NP-40, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA), and 1 ml of LiCl wash (250 mM LiCl, 1.0% NP-40, 0.5% SDC, 1 mM Na_2EDTA, 50 mM Tris-HCl, pH 8.0) to remove proteins non-specifically bound to the protein A agarose beads. The beads were then washed two times in 1 ml of TE buffer, pH 8.0.

xii. Western Blot Analysis of Immunoprecipitated Proteins

Protein A agarose beads bound to the anti-acetylated histone H3 antibody were resuspended in a small volume of TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) and SDS was added to a final concentration of 1.5% to release the protein-antibody complexes from the beads. The sample was then incubated at 68°C for 6 h to reverse the formaldehyde cross-links. Western blots were prepared by running the immunoprecipitated proteins on a SDS 15% polyacrylamide gel at 42 mA for 2.5 h. Proteins were then transferred from the SDS-polyacrylamide gel onto a nitrocellulose membrane (Bio-Rad, California) in the presence of CAPs transfer buffer (25 mM of 3-[cyclohexylamino]-1-propanesulfonic acid, pH 10, 20% methanol (v/v)). The protein transfer took place overnight at 50 V at 4°C. Following transfer, the membrane was incubated for 1 h at room temperature in a blocking solution containing 7.5% (w/v) skim milk and 0.2% (v/v) Tween-20 in 1X Tris-Buffered Saline (TBS: 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl). The membrane was then washed two times in 1X TBS containing 0.2% Tween-20, and immunochemically stained with the anti-

acetylated histone H3 antibody and the goat anti-rabbit antibody linked to horseradish peroxidase (Sigma, St. Louis, Missouri) using the ECL (enhanced chemiluminescence) detection system (Amersham Life Science Inc., Arlington Heights, Illinois).

xiii. Identification of DNA Cross-linked to Immunoprecipitated Proteins

Protein A agarose beads bound to the anti-acetylated H3 antibody were resuspended in 100 µl of TE buffer, pH 8.0. RNase A was then added to the protein A agarose-TE slurry, and the sample was incubated for 30 min at 37°C. Following this, proteinase K was added to the sample to a final concentration of 500 µg/ml, and the sample was incubated overnight at 37°C. The sample was then incubated at 68°C for 6 h to remove any residual cross-linked peptides from the DNA. At this point, the protein A beads were removed from the sample by centrifugation. Sodium acetate was then added to a final concentration of 0.3 M, and the sample was subjected to two phenol-chloroform-isoamyl alcohol (25:24:1) extractions, followed by precipitation with three volumes of 100% ethanol. In addition, DNA was isolated from an aliquot of the input and unbound chromatin under the same conditions used to isolate the histone H3-crosslinked DNA. 500 µg of input, unbound and bound DNA samples were made to 0.4 M NaOH and 10 mM Na_2EDTA, boiled for 10 min and then slot blotted onto a Hybond-N^+ nylon membrane (Amersham Life Science Inc.) using a Manifold™ II slot-blot manifold (Schleicher & Schuell, Keene, New Hampshire). All slots were subsequently washed with 0.4 M NaOH to fix the DNA on to the membrane.

xiv. Probe Preparation and Hybridization

Probes were labelled by random priming using the RadPrime DNA labeling System (Gibco, Grand Island, New York). The cloned DNA probe used was pCBG 14.4, an unique intronic sequence of the β-globin gene [68]. The probe was labeled to a specific activity of approximately 10^8 dpm/µg. Before, hybridization the slot-blot was incubated in aqueous pre-hybridization (APH) solution [5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 5X Denhardt solution, 1% (w/v) SDS, 100 µg/ml denatured salmon sperm DNA] for 1 h at 68°C. The probe (about 10 ng per ml of APH solution) was then denatured by boiling for

10 min, and then added to the slot blot along with fresh, pre-warmed APH solution. The hybridization took place overnight at 68°C, and was proceeded by 2- 5 min washes in 2X SSC / 0.1% SDS at room temperature, 2- 5 min washes in 0.2X SSC / 0.1% SDS at room temperature, 2- 15 min washed in pre-warmed (42°C) 0.2X SSC / 0.1% SDS for 15 min at 42°C, and, finally, one quick rinse in 2X SSC at room temperature.

B. ASSUMPTIONS

Our goal of year 1 of this project was to isolate transcriptionally active chromatin from human breast cancer T47D5 cells. In mammalian cells, histone H3 is the only histone with a cysteine. This cysteine residue in H3 at position 110 is buried in the interior of most nucleosomes and is not available to thiol reactive regents. The nucleosomes associated with transcribed DNA are unfolded and have the H3-cysteine exposed. The unique feature of transcribing chromatin is that their nucleosomes have a thiol-reactive H3.

The sulfhydryl group of histone H3 in transcriptionally active chromatin can covalently react with mercury and iodoacetyl group. We assumed that either organomercury columns or a newly developed technique using Sulfolink Coupling Gel could be used to isolate transcriptionally active chromatin. This gel consists of immobilized iodoacetyl on a cross-linked agarose support. Sulfolink gel covalently binds to thiol-reactive cysteine residues.

As transcriptionally active chromatin is associated with highly acetylated H3, we considered the CHIPs technology with anti-acetylated H3 antibodies as an alternate method to isolate transcriptionally active chromatin. We have initiated these studies with chicken erythrocyte chromatin. Our assumption is that the techniques developed with the erythrocyte system will be directly applicable to the isolation of transcriptionally active chromatin from breast cancer cells. Further as the CHIPs protocol uses formaldehyde cross-linking, the isolation of transcriptionally active chromatin bound to the nuclear matrix should be feasible.

C. RESULTS AND DISCUSSION

i. Restriction Endonuclease Digestion of Breast Cancer Chromatin

We have initially chosen the enzymes DpnII and MspI to fragment breast cancer chromatin. DpnII was chosen since this enzyme is commonly used to fragment DNA for RDA (representation difference analysis) analysis. The DpnII digestion buffer produced by the manufacturer to efficiently digest naked DNA consists of 100 mM NaCl, 50 mM Tris (pH 6.0), 10 mM $MgCl_2$, and 1 mM DTT (dithiothreitol). Although this buffer is efficient for digestion of naked DNA, its high $MgCl_2$ concentration and low pH may reduce the release of the chromatin fragments from nuclei. In a previous study performed by Workman and Langmore [69], $MgCl_2$ concentrations above 0.5 mM were shown to interfere with chromatin solubilization when chromatin was digested by various restriction enzymes. Based on these findings, we decided to use a digestion buffer containing a low $MgCl_2$ concentration that was described by these authors. Agarose gel electrophoresis of DNA from DpnII-digested chromatin displayed a pattern similar to the nucleosomal DNA ladder pattern generated by micrococcal nuclease digestion (Fig. 1 compare with Fig. 2). However, the bands generated with micrococcal nuclease were more diffuse than those produced with DpnII. Micrococcal nuclease preferentially digests linker DNA, that is the DNA between nucleosomes. We were concerned that endogenous endonucleases were cleaving the DNA. However, a mock digestion or digestion of the chromatin with the MspI restriction enzyme did not display the same pattern. Our conclusion was that under these conditions endogenous nuclease activity was suppressed. MspI did not appear to digest the chromatin to any significant extent. The generation of a repeat pattern with DpnII may be a consequence of the pH of the digestion buffer since DpnII may exhibit star (non-specific cutting) activity above a pH of 6.5. Alternatively, DpnII may cleave repetitive DNA, which would generate such a pattern. Nevertheless, these results show that DpnII can effectively cleave breast cancer chromatin. We will adjust the pH to 6.0 to investigate whether the DpnII-generated banding pattern is still produced.

ii. Fractionation of Chromatin from Human Breast Cancer T47D5 Cells

Our previous studies with chicken erythrocyte chromatin showed that the longevity of the

unfolded transcribing nucleosome was dependent upon time that the histones were maintained in highly acetylated states [48]. To maintain the highly acetylated state of the core histones (H2A, H2B, H3 and H4), we incubated breast cancer cells with sodium butyrate, a histone deacetylase inhibitor, before collecting the cells. We also analyzed cells that were not incubated with sodium butyrate.

During the course of these studies we found that the composition of the buffer to digest nuclei affected endogenous nuclease activity. Initially nuclei isolated from human breast cancer T47D5 cells were incubated with micrococcal nuclease (15 units/ml) at 37 ^{0}C for 10 min in Buffer B. During the course of digestion, chromatin fragments, typically mononucleosomes, will leak out of the nuclei. These chromatin fragments are collected in a fraction called SO. The nuclei are then resuspended in a low ionic strength buffer to release chromatin fragments from the nuclei. The fraction containing the soluble chromatin fragments is called SE, while the chromatin fragments remaining with the residual insoluble nuclear material is called fraction PE. Fraction PE contains chromatin fragments associated with the nuclear matrix. In the literature chromatin fraction PE is also referred to as the low-salt insoluble chromatin fraction [70]. Table I shows that the distribution of nuclear DNA (A_{260} or diphenylamine assay) among the chromatin fractions. In previous studies we found that measuring DNA content by absorbance at 260 nm was not reliable. We suspected that presence of RNA, which will also absorbs at 260 nm, or protein (cross-absorbance at 260 nm) made measurements at 260 nm unreliable [12]. Hence, we used the diphenylamine assay, which measures only DNA content. Table I shows that the content of DNA in the breast cancer chromatin fractions determined by A_{260} or diphenylamine assay were similar for some fractions (e.g., SO) but not for others (e.g., SE and PE). Cells incubated with or without sodium butyrate had similar distributions of DNA among the chromatin fractions.

DNA from the chromatin fractions was analysed by agarose gel electrophoresis, and the typical micrococcal nuclease digestion ladder can be observed (Fig. 2). The S0 fraction typically had mononucleosome length DNA (see lanes 1 and 10 in Fig. 2). DNA fragments isolated from fractions SE and PE either were processed to primarily the mononucleosome size DNA fragment (see lanes 2-5 in Fig. 2) or were a continuum of

sizes resolving a smear rather than a distinct banding pattern (lanes 11 and 12 in Fig. 2). These results are an indication of an endogenous nuclease activity [71].

On advisement from the study of Vanderbilt *et al.* [71], we subsequently changed our digestion buffer to TNM. Table I shows that when nuclei were digested in TNM there was a greater leakage of mononucleosomes into fraction S0 than there was with Buffer B (Fig. 2, lane 7). The TNM buffer has higher ionic strength than that of Buffer B, explaining the greater release of mononucleosomes into fraction S0 [72]. The distribution of DNA fragments among fractions SE and PE were similar when nuclei were digested in Buffer B or TNM (Table I). Fig. 2 (lane 8 and 9) shows the typical micrococcal nuclease digestion pattern for DNA fragments in fractions SE and PE. Also note that the lengths of the DNA fragments in fraction PE are greater than those in fraction SE. These results are typical of the relative DNA fragment lengths observed in previous studies [12].

The acid-soluble proteins extracted from chromatin fractions were analysed by AUT-PAGE. Fig. 3 shows that incubation of breast cancer cells for 2 h in sodium butyrate has a profound affect on the level of highly acetylated histone isoforms. The chromatin fractions S0, SE and PE have a low level of highly acetylated histones when isolated from cells not incubated with sodium butyrate (Fig. 3C). Histone H4 has low amounts of tri- and tetra-acetylated isoforms. However, when cells are incubated for 2 h with sodium butyrate, an increase in highly acetylated histones is readily seen in all chromatin fractions. Tri- and tetra-acetylated isoforms of H4 are seen in fractions S0, SE and PE (Fig. 3B). The digestion of nuclei in Buffer B or TNM did not affect the distribution of acetylated histone isoforms among the chromatin fractions S0, SE and PE.

Southern blotting was used to determine the distribution of transcriptionally active DNA sequences among the chromatin fractions. In T47D5 human breast cancer cells (estrogen-dependent, ER positive), ER and pS2 are transcriptionally active genes. DNA fragments from fractions S0, SE and PE were electrophoretically resolved on agarose gels and then transferred to membranes. The blots were probed with DNA fragments containing either ER cDNA or pS2 cDNA. Fig. 4 shows that fraction PE contained most of the transcriptionally active DNA sequences. These results are in agreement with our results with chicken erythrocyte chromatin where we found that most transcriptionally

active chromatin fragments were found with the low-salt insoluble chromatin fraction PE [12,73]. These results show that it is imperative that we succeed in isolating transcriptionally active chromatin from the nuclear matrix.

iii. Organomercury Column Chromatography

Based upon our results with chicken erythrocytes [48], we applied similar buffer systems and conditions to isolate the transcriptionally active chromatin by organomercury column chromatography. After testing of number of chromatin preparations from T47D5 cells, we found that the composition of the micrococcal nuclease digestion buffer affected the reactivity of the histone H3 thiol group. The exposed H3 thiol group of transcribing nucleosomes is susceptible to reaction with thiol reactive agents present in the buffer. Once the H3 thiol group has reacted with a thiol reactive agent, it will not be available for reaction with organomercury or iodoacetate. We encountered such problems when buffers containing thiodiglycol were used. The nuclei of T47D5 cells were digested with micrococcal nuclease in TNMT buffer, which contains thiodiglycol, and then, the fragmented chromatin was applied to the organomercury column. The proteins in mercury bound fraction were analysed by AUT- and SDS-PAGE (polyacrylamide gel electrophoresis). The mercury bound fraction did not contain core histones (H2A, H2B, H3, and H4). These studies showed that chromatin fragments prepared in this way do not bind to the organomercury column. When these experiments were repeated with buffers lacking thiodiglycol (Buffer B or TNM), binding of the chromatin fragments to the organomercury column was observed (see below). Although thiodiglycol does not have a reactive thiol group, we suspect that thiodiglycol preparation was contaminated with low levels of thiol reactive agents. We have since excluded thiodiglycol from our buffers.

The chromatin fragments in fractions SO and SE from micrococcal nuclease digested nuclei in buffer B were applied to the organomercury column. Following elution of the unbound chromatin fragments, the column was washed with 0.5 M NaCl to displace chromatin fragments bound to non-histone chromosomal proteins with thiol reactive groups [48]. Chromatin fragments bound to the organomercury column by thiol reactive H3 in unfolded nucleosomes were released by washing the column with a buffer containing DTT.

The unbound, 0.5 M NaCl eluted fraction and 10 mM DTT eluted fraction were collected and analysed. Table II shows the distribution of DNA fragments in the three fractions from the organomercury column. Most of the chromatin fragments in fractions S0 and SE did not bind to organomercury column. For the S0 fraction, about 75-80% of the chromatin did not bind, 13-18 percent DNA was washed from the column with 0.5 M salt, and only 5-6 percent DNA eluted from the column with DTT. With the SE fraction, the distribution of DNA in the column fractions was similar, with about 80% not binding and 3-6% being released from the column with DTT (see Table II). The amount of retained chromatin fragments is what we would expect considering the percent of chromatin that is engaged in transcription in a human breast cancer cell.

Fig. 5A shows the DNA fragments isolated from the organomercury column fractions resolved by agarose gel electrophoresis. The DTT released fraction from SO or SE was mononucleosome size DNA (lane 4 and 7 in Fig. 5A). For SE, the unbound and 0.5 M NaCl eluted chromatin had longer DNA fragments lengths than the DTT released chromatin. The histones extracted from the unbound, 0.5 M salt released and DTT released fractions were analysed by SDS- and AUT-PAGE. SDS-PAGE and AUT-PAGE show that organomercury-binding nucleosomes contain the four core histones (H2A, H2B, H3 and H4; see Fig. 5B, C). In this experiment, butyrate-treated T47D5 cells were used; the hyperacetylated H4 isoforms were observed in three fractions (Fig. 5C). The DTT released fractions were enriched high mobility group (HMG) proteins, which are non-histone chromosomal proteins with thiol reactive cysteines (lanes 4 and 8 in Fig. 5C).

Since PE contains most of the transcriptionally active chromatin, we were next going to fractionate the PE chromatin fraction by organomercury column chromatography. However, at this time the manufacturer of this column (BioRad) has discontinued this product. We thus designed a novel procedure that uses the Sulfolink column.

iv. Sulfolink Coupling Gel Chromatography

The Sulfolink Coupling Gel (Pierce) consists of immobilized iodoacetyl groups on a cross-linked agarose support. The Sulfolink iodoacetyl groups will react specifically with sulfhydryl groups.

To test its specificity towards thiol groups in human breast cancer chromatin, a cysteine blocking control study was performed. Cysteine will block the iodoacetyl groups in the Sulfolink agarose beads. The Sulfolink beads were incubated with 50 mM cysteine for 30 min. T47D5 chromatin fractions SE and PE were then incubated with cysteine-blocked or untreated beads. The percent of unbound chromatin from these beads was calculated by measuring the A_{260}. With fraction SE, 97-100% of the applied chromatin did not bind to blocked column, while about 80% was released from the untreated column (Table III). These results suggest that SE chromatin fragments will not be retained by the column when the iodoacetyl groups are blocked. When the column is not treated, we assume that chromatin fragments are being retained by thiol-reactive histone H3 of unfolded nucleosomes or by thiol-reactive non-histone chromosomal proteins reacting with the iodoacetyl group.

For fraction PE chromatin, 84% of the DNA was released from the cysteine-blocked beads, while 69% was released from the untreated beads. These results suggest that PE chromatin is being bound to the column by thiol reactive proteins. However, the binding of PE chromatin to the blocked column is of concern. If chromatin fragment size is causing the long fragments to be trapped in the gel matrix, then Sulfolink column chromatography may not be applicable to the fractionation of PE chromatin. However, as the DNA was measured by A_{260}, the apparent distribution of DNA may be incorrect. This study will be repeated and DNA concentration will be determined by the diphenylamine assay. Further, we will alter the buffer and conditions by which the column is loaded with PE chromatin and subsequently washed.

Chromatin fragments may be retained by the Sulfolink column indirectly by non-histone chromosomal proteins that have reacted with the iodoacetyl group or directly by the thiol-reactive H3 of unfolded nucleosomes. To break contacts between thiol-reactive non-histone chromosomal proteins and chromatin fragments, we washed the column with 0.8 M NaCl. Table IV shows that 88, 82 and 74% of the chromatin of fractions S0, SE and PE, respectively, applied to the column were present in the unbound and 0.8 M NaCl wash fractions. The DNA of the chromatin fragments remaining bound after the salt wash was released by digesting the beads with proteinase K, and then the DNA was isolated. Fig.

6 shows the DNA fragment lengths present in the various column fractions. For the S0 fraction, the bound fraction consisted of mononucleosomes (Fig. 6, lane 5). For the SE fraction, the unbound fraction contained oligonuclesomes (Fig. 6, lane 7), and the bound fraction consisted of shorter mono- and di-nucleosomes (Fig. 6, lane 9). This result suggests that longer chromatin fragments are not being trapped by the column matrix. As micrococcal nuclease processes transcriptionally active chromatin faster than bulk chromatin, finding that shorter chromatin fragments are retained by the column is consistent with the retention of transcriptionally active chromatin [12].

For PE chromatin, Fig. 6 (lane 13) shows that bound fragments were of similar size to those released from the column. Two results of column chromatography are encouraging. First, compared to fractions S0 and SE, a greater percentage of PE chromatin fragments was bound to the column. As PE chromatin is enriched in transcriptionally active DNA, this result is expected. Second, transcriptionally active chromatin fragments associated with the nuclear matrix are longer than those released into S0 and SE [12]. Southern blot analysis of these fractions for the presence of transcriptionally active and repressed DNA sequences will determine how well the column is operating in the isolation of transcriptionally active chromatin.

The strategy for releasing chromatin and DNA fragments from the Sulfolink column has been revised. Following the addition of chromatin fragments from fractions S0 and SE, the column was sequentially washed with buffers containing 0.8 M, 1.2 M and 2 M NaCl. The rationale for choosing these salt concentrations is as follows. As stated previously, the 0.8 M NaCl wash would remove chromatin bound to the column indirectly by binding to non-histone chromosomal proteins. Previous studies indicated that unfolded nucleosomes dissociated at 1.2 M NaCl [48]. Thus, a 1.2 M NaCl wash may release transcriptionally active DNA from the column bound H3. The final 2 M NaCl wash will dissociate remaining DNA from column bound H3. Table V summarizes the DNA distribution among the column fractions from S0 and SE attached to the Sulfolink column. For the S0 fraction, 5 and 2% of the input DNA was found in the 1.2 and 2 M NaCl extracted fractions, respectively. However, we note that the diphenylamine assay results are quite different form the A_{260} measurements. Typically diphenylamine and A_{260}

measurements are similar for this fraction. Thus, this data set needs repeating. For the SE fraction, 14 and 7% of the input DNA was found in the 1.2 and 2 M NaCl extracted fractions, respectively.

The DNA fragments isolated from the Sulfolink column with SE chromatin fragments were electrophoretically resolved by agarose gel electrophoresis and transferred to membranes for Southern blot analysis. We studied the distribution of three transcriptionally active DNA sequences, c-*myc*, pS2, and ER, and one repressed DNA sequence, apolipoprotein 5' matrix attachment region (MAR). Fig. 7 shows the transcriptionally active DNA sequences (ER, ps2 and c-myc) were present in the 0.8, 1.2 and 2 M NaCl fractions. However, we repeatedly observed an increase abundance of these sequences in the 1.2 and 2 M NaCl extracted fractions. In contrast, repressed DNA sequences were poorly represented in the 0.8, 1.2 and 2 M NaCl fractions. These results provide strong evidence that the novel Sulfolink column strategy that we have devised to isolate transcriptionally active chromatin is feasible.

To analyze the histones, SE chromatin was applied to the Sulfolink column, which was subsequently washed with a buffer containing 0.8 M NaCl. The histones in these fractions and those remaining bound to the column were isolated by acid extraction, TCA precipitated, and electrophoresed on AUT polyacrylamide gels. The AUT gels were Coomassie blue stained or transferred onto a nitrocellulose filter for Western blotting. Fig. 8A shows a Coomassie blue stained gel where the Sulfolink bound fractions were enriched in hyperacetylated H3 and H4 isoforms. This was confirmed by Western blot analysis using anti-acetylated H3 (Fig. 8B) and anti-acetylated H4 antibodies (Fig. 8C). It is important to note the striking enrichment of highly acetylated H3 in the bound fraction. Because some of the thiol reactive H3 may remain bound to the Sulfolink column, we believe that the true enrichment of highly acetylated H3 in this fraction is an underestimate. These observations agree with our previous studies with chicken erythrocyte chromatin that H3-thiol reactive nucleosomes are associated with highly acetylated H3 [48].

Our results from 13 Sulfolink column chromatography experiments indicate that the Sulfolink column can capture transcriptionally active chromatin with thiol-reactive histone H3, and the Sulfolink Coupling Gel column method can isolate transcriptionally active

genomic DNA from human breast cancer cells.

v. CHIPs Strategy to Isolate Transcriptionally Active Chromatin

An alternate method to isolate transcriptionally active chromatin is to exploit the observation that transcribed, but not repressed, chromatin is associated with highly acetylated H3. Our plan is to apply the CHIPs technology to isolate transcriptionally active chromatin from breast cancer cells. In brief nuclei are incubated briefly with formaldehyde to cross-link highly acetylated H3 to transcriptionally active DNA. Interestingly the formaldehyde treatment appears to dissociate chromatin from the nuclear matrix [74]. The chromatin is fragmented by restriction endonuclease, sonication or micrococcal nuclease digestion. A dissociation buffer is applied, which solubilizes 90% or greater of the cross-linked chromatin. The chromatin fragments with highly acetylated H3 are isolated by immunoprecipitation with an antibody that with very high specificity recognizes highly acetylated H3. Transcriptionally active DNA is isolated from the immunoprecipitated chromatin. We have initiated these studies by working out conditions with a chromatin source familiar to us, chicken immature erythrocytes [48].

The extent of formaldehyde cross-linking can influence the release of DNA from the nucleus [75]. To obtain a reasonably sized DNA pool from which to work, it is necessary to obtain maximum DNA yields. Nuclei isolated from chicken immature erythrocytes were treated with 1% formaldehyde for varying times, and then the nuclei were placed in lysis buffer. The cross-linked chromatin that was soluble following centrifugation was determined. Table VI displays the percent of DNA released from the sonicated nuclei cross-linked with formaldehyde for various times. These values are based on A_{260} values of DNA in the sonicated nuclear lysate cleared of insoluble nuclear material relative to values in the complete nuclear lysate. Over 96% of the nuclear DNA was released by sonication when nuclei were cross-linked with formaldehyde from 0 to 10 min. However, the amount of released DNA decreased when the time of formaldehyde cross-linking exceeded 10 min.

To determine that highly acetylated H3 was being cross-linked to DNA, we devised a novel strategy by which chromatin fragments liberated from sonicated nuclei in lysis

buffer were applied to a HAP column. The HAP column was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA, which was bound to the HAP column. To reverse the cross-links the HAP slurry was heated to 68^{0}C. The released proteins were analyzed in Western blotting experiments with anti-acetylated H3 antibodies. As a control, the same amount of sonicated nuclei not treated with formaldehyde and disrupted in lysis buffer was applied to the HAP column. Fig. 9 shows that highly acetylated H3 isoforms were cross-linked to DNA. The extent of the cross-linking of highly acetylated H3 to DNA could not be determined from this figure due to the saturation of the chemiluminescence signal. The lane displaying HAP-bound proteins not cross-linked to DNA (the control) displays a high level of highly acetylated H3 only because equal amounts of protein were loaded for each sample. All protein samples were resuspended in equal volumes of urea following lyophilization. The amount of protein recovered from the cross-linked sample was at least five times greater than that of the non-cross-linked samples. This study is being repeated by loading equal volumes from the HAP column and using shorter times for immunochemical staining. By comparing the amount of highly acetylated H3 in the input sample versus the amount of highly acetylated H3 cross-linked to DNA, we will know the amount of acetylated H3 that is cross-linked to DNA.

The Western blot analysis also showed the presence of H3 dimers. It appears that the conditions for cross-link reversal are not sufficient to reverse the protein-protein cross-links. Heating the protein samples at 100°C in the presence of 2 M guanidine-HCl and 0.5 M 2-mercaptoethanol for 30 min will likely reverse protein-protein cross-links. Such conditions have been shown to completely remove formaldehyde-induced protein-protein cross-links [76]. Based on the DNA release values and Western blot analysis data, we concluded that a cross-linking duration of 5 to 10 min was sufficient to efficiently cross-link highly acetylated H3 to DNA without encountering the problems associated with extensive protein-DNA and protein-protein cross-linking.

To familiarise ourselves with the CHIPs technology, we used the S150 chromatin fraction from immature chicken erythrocytes, which were incubated with 10 mM sodium butyrate for 60 min to drive dynamically acetylated histones into their highest acetylated isoforms [48,73]. The chromatin fragments in this fraction were formaldehyde cross-linked

and immunoprecipitated with anti-acetylated H3 antibody. Western blot analysis of the proteins isolated from the immunoprecipitation procedure indicates that, in both cross-linked and non-cross-linked S150 fractions, the highly acetylated histone H3 isoforms were immunoprecipitated with the anti-acetylated H3 antibody in conjunction with the protein A agarose beads (Fig. 10). Moreover, cross-linked or non-cross-linked highly acetylated histone H3 does not associate with the protein A agarose beads in a manner independent of the anti-acetylated H3 antibody (Fig. 10, lanes 3 and 5).

Following protein analysis, the β-globin intronic sequence was hybridized as a radio-labelled probe to a slot-blot containing DNA isolated from the S150 input chromatin, the S150 input chromatin not immunoprecipitated by the anti-acetylated antibody, and the S150 input chromatin immunoprecipitated by the anti-acetylated antibody (Fig. 11). The resulting autoradiogram shows that the transcriptionally active chicken β-globin DNA sequences are associated with highly acetylated H3. The presence of β-globin DNA sequences in the chromatin not immunoprecipitated with the anti-acetylated H3 chromatin is a combined measure of the cross-linking and immunoprecipitation efficiencies. Although our results are similar to those of others [77], clearly the cross-linking and immunoprecipitation efficiency could be improved. The novel protocols that we have designed in monitoring cross-linking efficiencies should aid us in revising conditions.

D. RECOMMENDATIONS

The progress made this past year is on target with our proposed Statement of Work (see page 9). We are confident that in the next two months we will have isolated transcriptionally active chromatin from human breast cancer T47D5 cells. We will continue to pursue the novel Sulfolink column chromatography procedure but with chromatin fragmented with DpnII. However, if we cannot successfully isolate DpnII-cleaved fragments from fraction PE, then this strategy may be discontinued. In the meantime we will apply the CHIPs technology with anti-acetylated H3 antibodies to isolate transcriptionally active chromatin. We have developed novel protocols to determine cross-linking efficiency. These procedures will be applied to the isolation of transcriptionally active chromatin from human breast cancer cell nuclei that have been digested with DpnII.

We currently obtain the anti-acetylated H3 antibody from Dr. David Allis. However, it is also commercially available, and we are considering preparing large amounts of the antibody ourselves.

7. CONCLUSIONS

Transcriptionally active chromatin has two features that distinguish it from bulk chromatin: unfolded nucleosomes with thiol reactive H3 and highly acetylated H3. We have exploited both of these features to isolate transcriptionally active chromatin. A novel Sulfolink column chromatography procedure has been developed for the isolation of transcriptionally active chromatin from human breast cancer cells. Further, we are applying a chromatin immunoprecipitation assay with anti-acetylated histone H3 antibodies as an alternate method to isolate transcriptionally active chromatin. Genomic DNA isolated from transcriptionally active chromatin should contain all genes transcribed in human breast cancer cells, including those that produce strictly nuclear RNAs.

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9. APPENDICES

Fig. 1. Restriction enzyme cleavage of chromatin in T47D5 human breast cancer cells. Nuclei were digested with either DpnII or MspI for 3 h at 37°C and 1 μg of DNA was electrophoresed on a 0.8% agarose gel. Lane 1. Low DNA Mass Ladder starting at 2000 bp at the top of the gel, followed by 1200 bp, 800 bp, 400 bp, 200 bp, and finally, 100 bp fragments (GibcoBRL, Gaithersburg, MD). Lane 2. DNA from nuclei resuspended in digestion buffer and incubated for 3 h at 37°C. Lane 3. DNA from nuclei digested with 2 U DpnII/μg DNA. Lane 4. DNA from nuclei digested with 5 U DpnII/μg DNA. Lane 5. DNA from nuclei digested with 2 U MspI/μg DNA. Lane 6. DNA from nuclei digested with 5U DpnII/μg DNA.

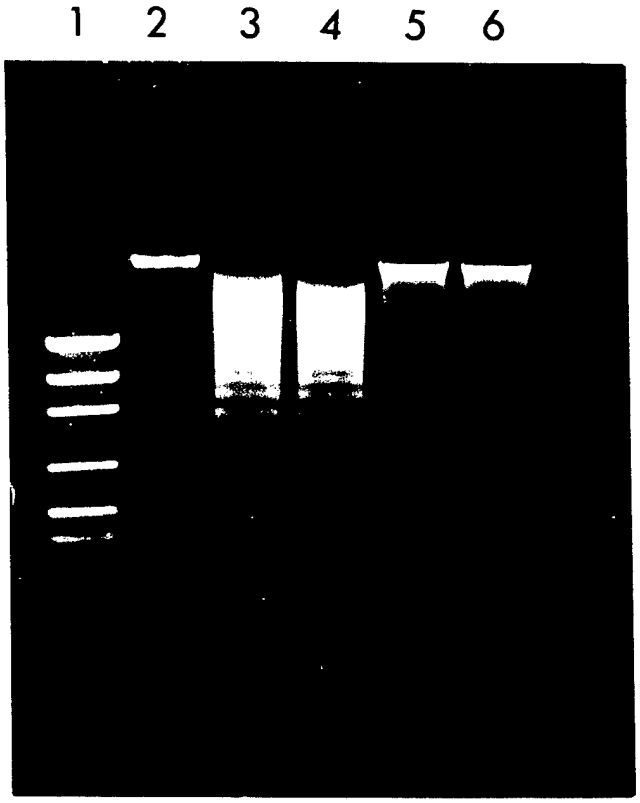

Fig. 2. Analysis of DNA fragments from fractionated human breast cancer chromatin. T47D5 breast cancer cells were incubated with 10 mM sodium butyrate for 2 h to inhibit deacetylation of highly acetylated histones. The nuclei were digested either in Buffer B (lanes 1-5 and 10-12) or TNM (lanes 7-9). The chromatin was fractionated as described under Experimental Procedures (see pg. 10), yielding fractions SO, SE and PE. Unless stated otherwise each lane had 10 μg of DNA. Lanes 1, 7 and 10 contain DNA fragments from fraction S0. Lanes 2, 3 (15 μg), 8, and 11 contain DNA fragments from fraction SE. Lanes 4, 5 (15 μg), 9, and 12 contain DNA fragments from chromatin fraction PE. Lanes 6 and 13 contain 1 kb DNA ladder (BRL). The DNA fragments were electrophoretically resolved on a 0.8% agarose gel and then visualized by ethidium bromide staining.

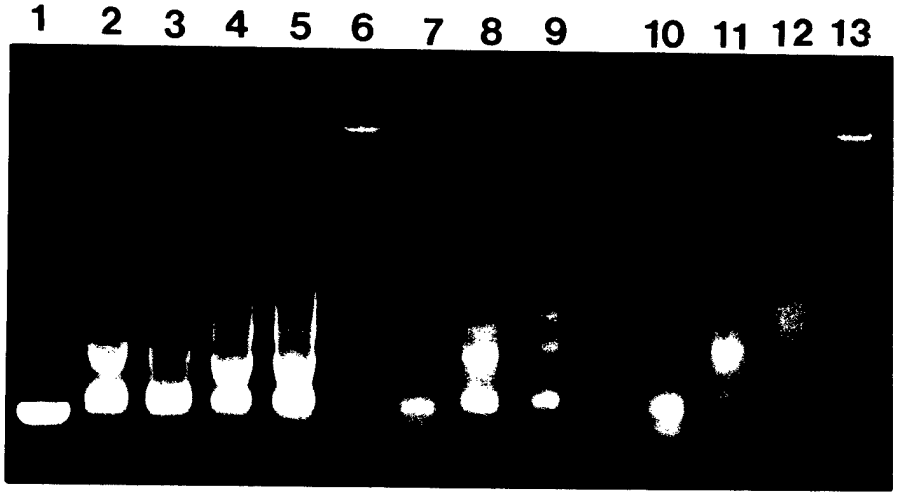

Fig. 3. Analysis of histones from fractionated human breast cancer chromatin. T47D5 breast cancer cells were incubated without (panel C) or with (panel A and B) 10 mM sodium butyrate (Na.B) for 2 h to inhibit deacetylation of highly acetylated histones. The nuclei were digested either in Buffer B (panel B) or TNM (panel A and C). The chromatin was fractionated as described under Experimental Procedures (see pg. 10), yielding fractions SO, SE and PE. Panel A: Each lane had 10 μg of DNA fragments, which were electrophoretically resolved on a 0.8% agarose gel and then visualized by ethidium bromide staining. Panel B and C: Each lane had 10 μg of protein, which was resolved by AUT 15% PAGE, and then the proteins were visualized by Coomassie Blue staining. Lanes 1, 2 and 3 contain either DNA or histones from fractions S0, SE and PE, respectively. The numbers 0, 1, 2, 3, and 4 point to the position of un-, mono-, di, tri-, and tetra-acetylated isoforms of H4.

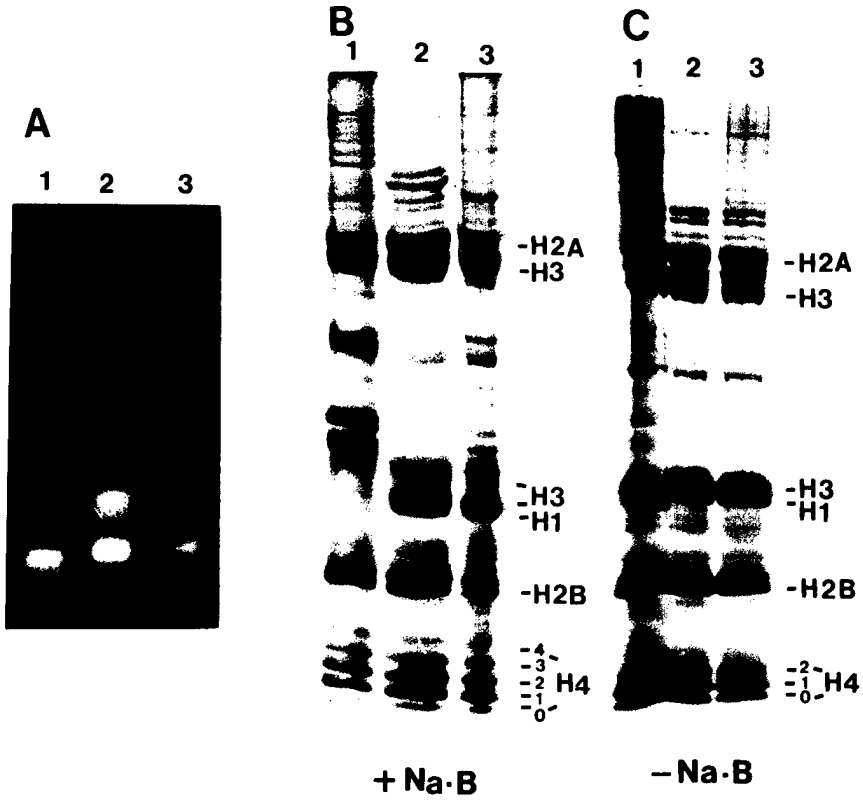

Fig. 4. Southern blot analysis of chromatin fractions from T47D5 human breast cancer cells. T47D5 breast cancer cells were incubated with 10 mM sodium butyrate for 2 h to inhibit deacetylation of highly acetylated histones. The nuclei were digested in Buffer B. The chromatin was fractionated as described under Experimental Procedures (see pg. 10), yielding fractions SO, SE and PE. Panel A and C: Each lane had 10 μg of DNA fragments, which were electrophoretically resolved on a 0.8% agarose gel and then visualized by ethidium bromide staining. Panel B and D: The DNA fragments were transferred to membranes, which were hybridized to ER cDNA (panel B) or pS2 cDNA (panel D) radiolabeled probes. The autoradiograms are shown. Lanes 1, 2 and 3 contain DNA fragments from fractions S0, SE and PE, respectively.

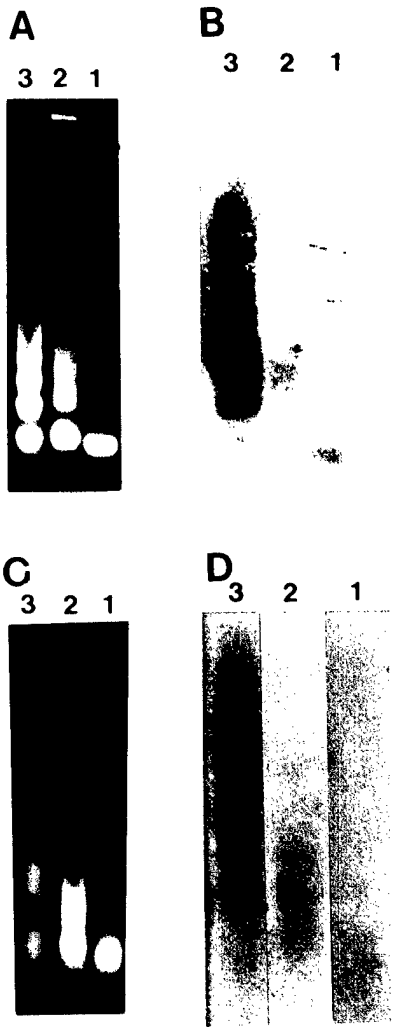

Fig. 5. Organomercury column chromatography of T47D5 human breast cancer chromatin. T47D5 breast cancer cells were incubated with 10 mM sodium butyrate for 2 h to inhibit deacetylation of highly acetylated histones. The nuclei were digested in Buffer B. The chromatin was fractionated as described under Experimental Procedures (see pg. 10), yielding fractions SO, SE and PE. Panel A: Each lane had 5 μg of DNA fragments, which were electrophoretically resolved on a 0.8% agarose gel and then visualized by ethidium bromide staining. Lane 1 contains the 1 kb DNA ladder marker. Lanes 2 and 5 have input DNA fragments from fractions S0 and SE, respectively. Lanes 3 and 6 have DNA fragments from the 0.5 M NaCl wash from fractions S0 and SE, respectively. Lanes 4 and 7 have DNA fragments from the DTT released fractions from S0 and SE chromatin, respectively. Panel B and C: Proteins from the input and column fractions were resolved by SDS 15% PAGE (panel B) or AUT 15% PAGE (panel C), and then the proteins were visualized by Coomassie Blue staining. The proteins were loaded by volume. Panel B: Lanes 1 (0.1 ml) and 5 (0.02 ml) have proteins from fractions S0 and SE, respectively. Lanes 2 (0.3 ml) and 6 (0.2 ml) have proteins from the unbound fractions from S0 and SE, respectively. Lanes 3 (1 ml) and 7 (1 ml) have proteins from the 0.5 M NaCl wash from fractions S0 and SE, respectively. Lanes 4 (2 ml) and 8 (2 ml) have proteins from the DTT released fractions from S0 and SE chromatin, respectively. Panel C: Lanes 1 (0.1 ml) and 5 (0.03 ml) have proteins from fractions S0 and SE, respectively. Lanes 2 (0.1 ml) and 6 (0.025 ml) have proteins from the unbound fractions from S0 and SE, respectively. Lanes 3 (1 ml) and 7 (0.5 ml) have proteins from the 0.5 M NaCl wash from fractions S0 and SE, respectively. Lanes 4 (2 ml) and 8 (2 ml) have proteins from the DTT released fractions from S0 and SE chromatin, respectively. The numbers 0, 1, 2, 3, and 4 point to the position of un-, mono-, di, tri-, and tetra-acetylated isoforms of H4.

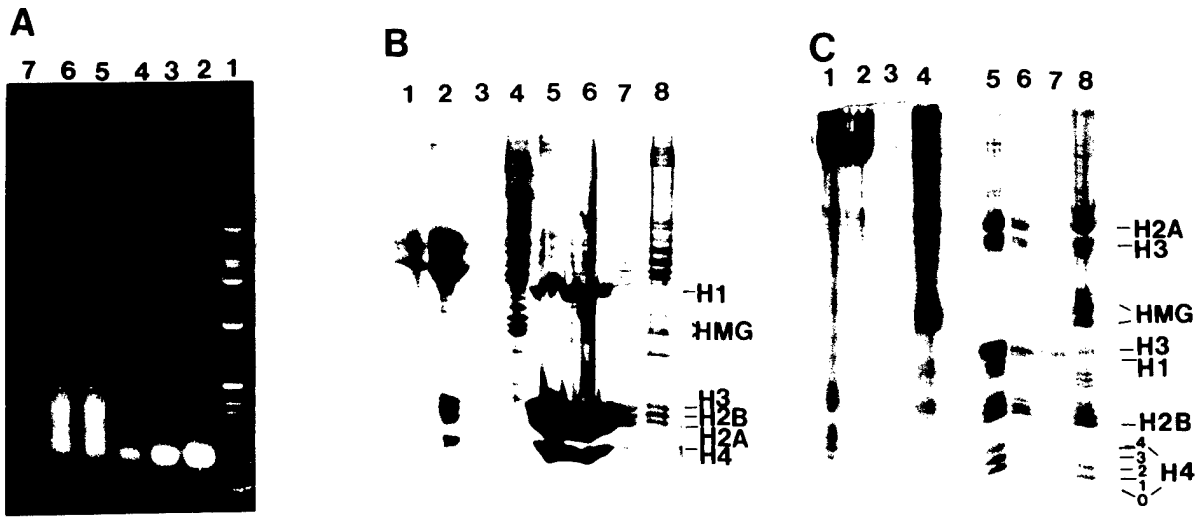

Fig. 6. Sulfolink column chromatography of T47D5 human breast cancer chromatin. T47D5 breast cancer cells were incubated with 10 mM sodium butyrate for 2 h to inhibit deacetylation of highly acetylated histones. The nuclei were digested in TNM. The chromatin was fractionated as described under Experimental Procedures (see pg. 10), yielding fractions SO, SE and PE. Panel A: Each lane had 8 μg of DNA fragments, which were electrophoretically resolved on a 0.8% agarose gel and then visualized by ethidium bromide staining. Lane 1 contains the 1 kb DNA ladder marker. Lanes 3, 7 and 11 contain DNA fragments not bound to the column with S0, SE and PE chromatin, respectively. Note that the S0 unbound fraction was lost during this run. Lanes 4, 8 and 12 contain DNA fragments from S0, SE and PE chromatin, respectively. Lanes 5, 9 and 13 contain DNA fragments, which were isolated from the column by proteinase K, phenol/isoamyl alcohol extraction and ethanol precipitation, from S0, SE and PE chromatin, respectively. Note that lane 5 was underloaded.

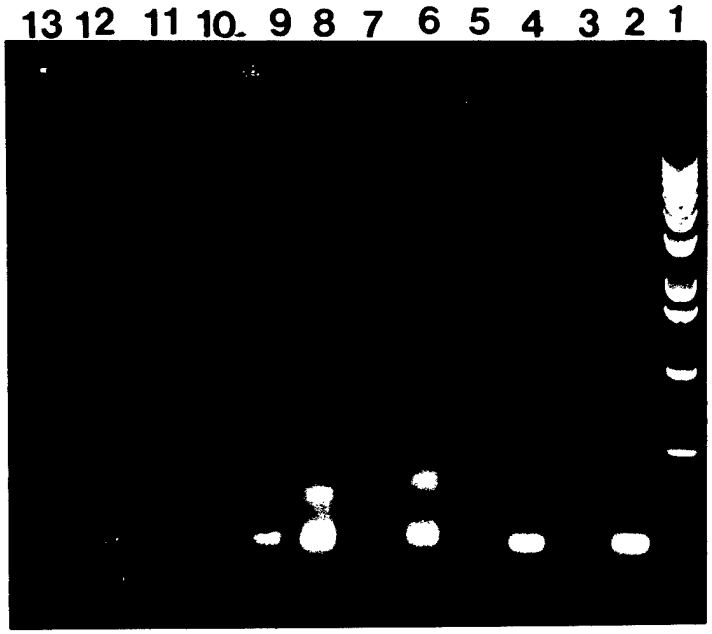

Fig. 7. Analysis of SE chromatin from T47D5 human breast cancer cells fractionated by Sulfolink column chromatography. T47D5 breast cancer cells were incubated with 10 mM sodium butyrate for 2 h to inhibit deacetylation of highly acetylated histones. The nuclei were digested in TNM. The chromatin was fractionated as described under Experimental Procedures (see pg. 10), yielding fractions SO, SE and PE. Panel A: Each lane had 10 μg of DNA fragments, which were electrophoretically resolved on a 0.8% agarose gel and then visualized by ethidium bromide staining. Panels B-E: The DNA fragments were transferred to membranes, which were hybridized to a genomic c-*myc* probe (panel B), genomic ER probe (panel C), pS2 cDNA probe (panel D), or apolipoprotein A1 5' MAR (MAR) genomic probe (panel E). The autoradiograms are shown in panels B-D. Lanes 1-5 contain DNA fragments from input SE chromatin fragments, chromatin fragments not bound to the column, 0.8 M NaCl wash of the column, 1.2 M NaCl wash of the column, and 2 M NaCl wash of the column, respectively.

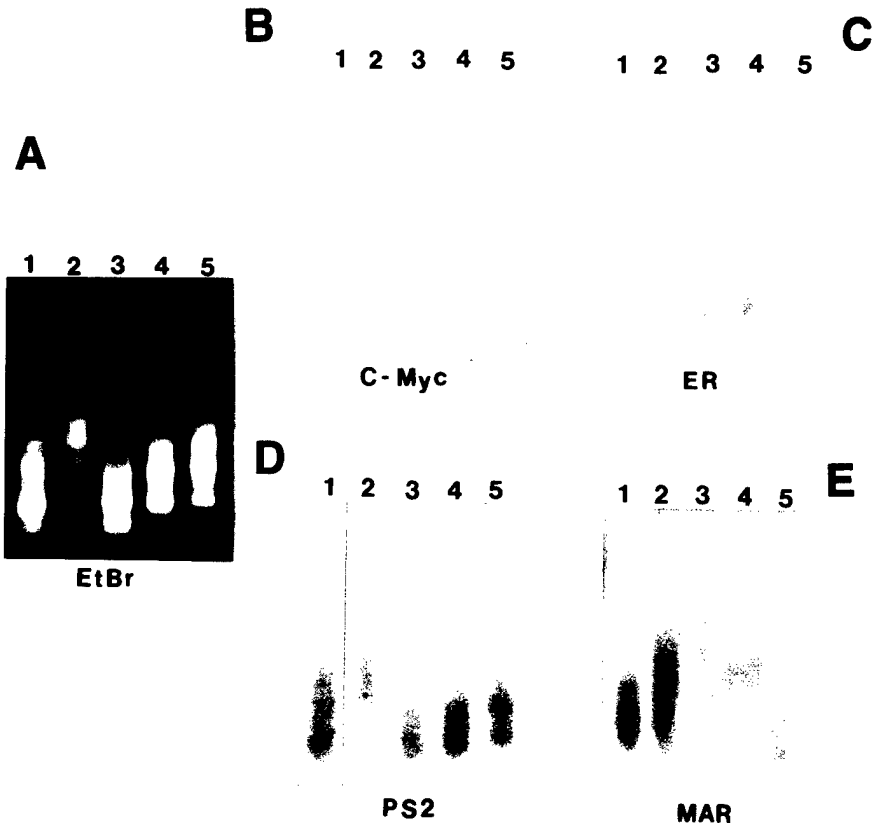

Fig. 8. Analysis of histones from Sulfolink column fractionated SE chromatin of T47D5 human breast cancer cells. T47D5 breast cancer cells were incubated with 10 mM sodium butyrate for 2 h to inhibit deacetylation of highly acetylated histones. The nuclei were digested in Buffer B. The chromatin was fractionated as described under Experimental Procedures (see pg. 10), yielding fractions SO, SE and PE. The SE chromatin was applied to a Sulfolink column. Histones were isolated by acid extraction from the unbound chromatin (lane 1), chromatin fragments in the 0.8 M NaCl wash (lane 2), and the chromatin fragments remaining bound to the column (lane 3). Each lane had 10 μg of protein. The histones were resolved by AUT 15% PAGE, and the gel was subsequently stained with Coomassie Blue (panel A) or transferred to nitrocellulose membranes and immunochemically stained with anti-acetylated H3 (panel B) or anti-acetylated H4 (panel C) antibodies. Variants of H3, H3.2 and H3.3 are shown. The numbers 0, 1, 2, 3, and 4 point to the position of un-, mono-, di, tri-, and tetra-acetylated isoforms of H4.

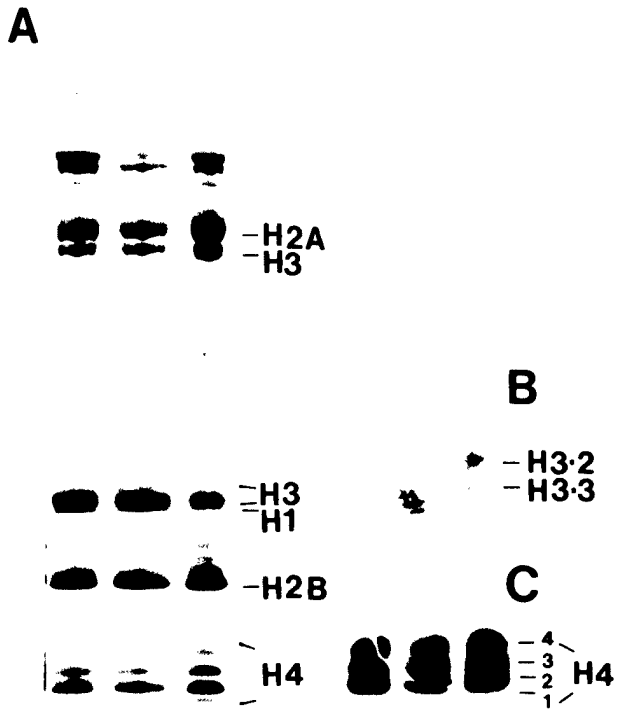

Fig. 9. Western blot analysis of formaldehyde-crosslinked proteins isolated by hydroxyapatite chromatography of chicken erythrocyte chromatin. Cross-linked and non-cross-linked chromatin fragments were bound to HAP as described under "Experimental procedures" (see page 14). Ten μg of protein was resolved by 5% SDS PAGE. The proteins were transferred to a nitrocellulose membrane and immunochemically stained with anti-acetylated H3 antibody. Lane 1. Acid-extracted histones from immature chicken erythrocyte nuclei. Lane 2. Proteins bound to the HAP without being cross-linked to DNA. Lane 3. Proteins cross-linked to DNA by formaldehyde over 5 min. Lane 4. Proteins cross-linked to DNA by formaldehyde over 10 min. Lane 5. Proteins cross-linked to DNA by formaldehyde over 14 min. Lane 6. Proteins cross-linked to DNA by formaldehyde over 60 min. AcH3 designates the position of highly acetylated H3. Unlabelled arrows mark the positions of acetylated H3 dimers.

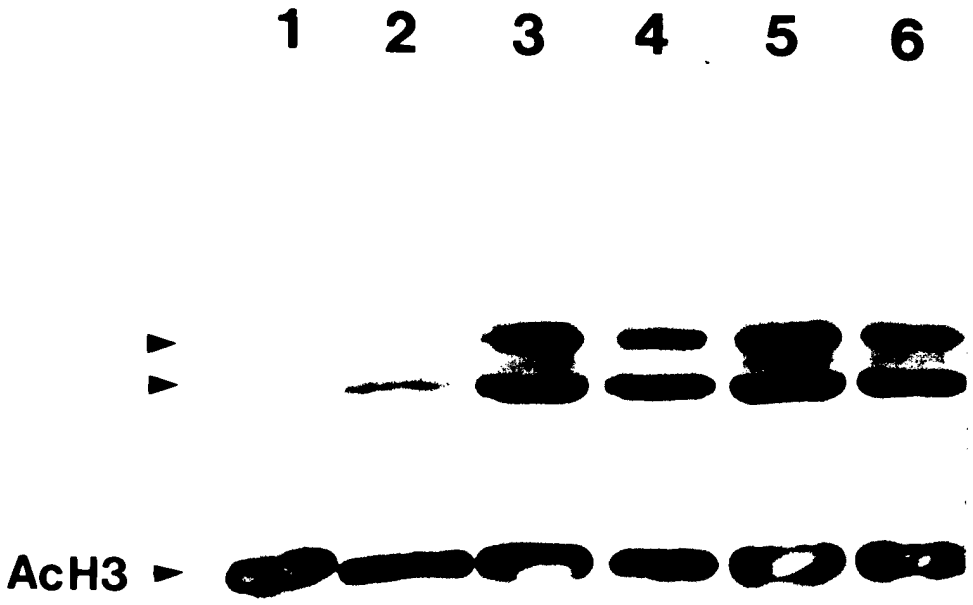

Fig. 10. Western blot analysis of chicken erythrocyte proteins recovered after immunoprecipitation. S150 chromatin fragments cross-linked with formaldehyde were incubated in the presence or absence of anti-acetylated H3 antibody, conjugated to protein A agarose, and collected by incubation with 1.5% SDS for Western blot analysis with anti-acetylated H3 antibody. Equal volumes of each preparation were loaded in their respective lane. Lane 1. Formaldehyde-cross-linked proteins isolated by the incubation of S150 chromatin fragments with antibody and then protein A agarose and immunochemically stained only with secondary antibody. Lane 2. Proteins isolated by the incubation of S150 chromatin fragments with antibody and then protein A agarose. Lane 3. Proteins isolated by the incubation of S150 chromatin with only protein A agarose. Lane 4. Formaldehyde-cross-linked proteins isolated by the incubation of S150 chromatin fragments with antibody and then protein A agarose. Lane 5. Formaldehyde-cross-linked proteins isolated by the incubation of S150 chromatin with only protein A agarose. Lane 6. Acid-extracted histones from immature chicken erythrocytes (1 μg). AcH3 designates the position of highly acetylated H3.

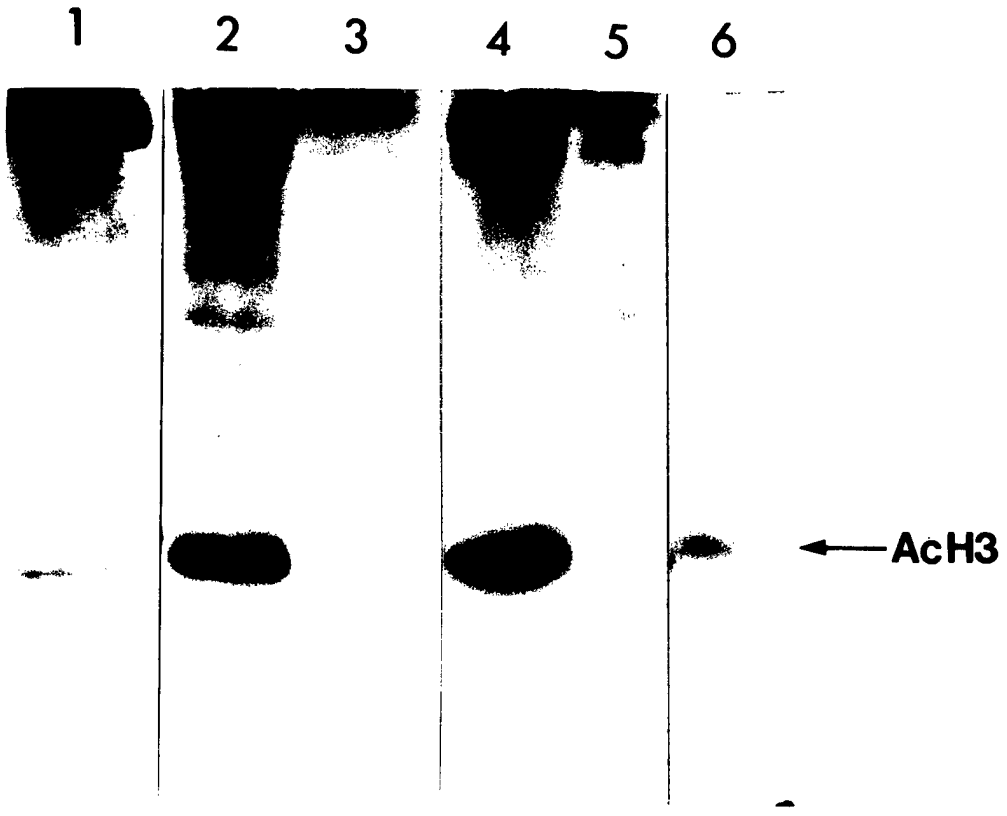

Fig. 11. Southern slot-blot analysis of DNA after immunoprecipitation. DNA fragments were isolated from input, unbound and bound fractions of S150 formaldehyde-cross-linked chromatin after immunoprecipitation with an anti-acetylated H3 antibody. DNA (500 ng) from each fraction was slotted onto a Hybond N^+ nylon membrane (Amersham) and hybridized to an intronic sequence from the β-globin gene. Slot 1. Input DNA. Slot 2. DNA from chromatin not immunoprecipitated with the antibody. Slot 3. DNA from chromatin immunoprecipitated with antibody.

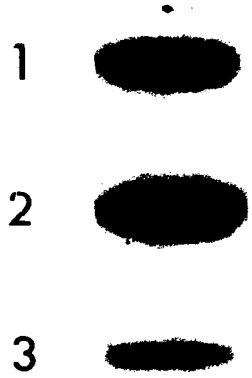

Table I. Distribution of DNA among chromatin fractions from T47D5 human breast cancer cells. Nuclei were digested in Buffer B or TNM. The DNA was measured by absorbance at 260 nm or by the diphenylamine assay. The values shown are fractions of total nuclear DNA.

	Buffer B		TNM	
Fraction	A_{260} (n = 6)	Diphenylamine (n = 2)	A_{260} (n = 5)	Diphenylamine (n = 2)
S0	0.16 ± .02	0.18 ± .01	0.27 ± .04	0.25 ± .01
SE	0.35 ± .05	0.50 ± .03	0.33 ± .07	0.41 ± .03
PE	0.46 ± .06	0.31 ± .02	0.40 ± .04	0.34 ± .03

Table II. Distribution of DNA among chromatin fractions chromatographed on an organomercury column. Nuclei were digested in Buffer B or TNM. The DNA was measured by absorbance at 260 nm or by the diphenylamine assay. The values shown are fractions of input DNA.

	Chromatin fraction S0		Chromatin fraction SE	
Fraction	A_{260} (n = 2)	Diphenylamine	A_{260} (n = 2)	Diphenylamine
Unbound	0.83 ± .05	0.76	0.87 ± .01	0.89
0.5 M NaCl wash	0.13 ± .03	0.18	0.10 ± .02	0.04
DTT released	0.05 ± .01	0.06	0.03 ± .01	0.06

Table III. Distribution of DNA following addition of breast cancer chromatin fractions to untreated and cysteine-treated Sulfolink column. SE and PE chromatin fractions were applied to a Sulfolink column that was either untreated or treated with cysteine to react with iodoacetyl groups. The fraction of input chromatin eluting from the columns is indicated. DNA was measured by absorbance at 260 nm.

Treatment	Chromatin fraction SE	Chromatin fraction PE
cysteine block	0.97 ± .04 (n = 3)	0.84 ± .04 (n = 2)
untreated	0.79 ± .05 (n = 2)	0.69 ± .02 (n = 2)

Table IV. Distribution of DNA following fractionation of chromatin fractions S0, SE and PE by Sulfolink chromatography. The chromatin fractions were applied to the Sulfolink. Following the elution of unbound chromatin, the column was washed with 0.8 M NaCl. The precentage of input chromatin in the unbound and 0.8 M NaCl wash is shown. The DNA content was measured by absorbance at 260 nm or by the diphenylamine assay.

Fraction	A_{260}	Diphenylamine
S0	82	88
SE	80	82
PE	77	74

Table V. Distribution of DNA among column fractions from a Sulfolink column. S0 and Se chromatin from T47D5 human breast cancer cells were applied to a Sulfolink column. Following removal of unbound chromatin, the column was sequentially washed with 0.8, 1.2 and 2.0 M NaCl. The DNA in each fraction was measured by absorbance at 260 nm or diphenylamine assay.

	Chromatin fraction S0		Chromatin fraction SE	
Fraction	A_{260} (n = 5)	Diphenylamine (n = 2)	A_{260} (n = 5)	Diphenylamine (n = 1)
unbound	0.81 ± .05	0.51 ± .07	0.56 ± .07	0.46
0.8 M NaCl	0.12 ± .03	0.22 ± .05	0.22 ± .07	0.32
1.2 M NaCl	0.05 ± .01	0.14 ± .03	0.14 ± .03	0.14
2.0 M NaCl	0.02 ± .01	0.11 ± .01	0.08 ± .01	0.07

Table VI. Percent DNA release from sonicated immature chicken erythrocytes treated with 1% formaldehyde for various lengths of time.

Time of Crosslinking	Percent DNA
0	96.3
5	96.9
10	98.5
14	82.1
60	36.5